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Oxidative stress increases 1-deoxysphingolipid levels in chronic kidney disease

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Abbreviations:

CKD, Chronic kidney disease; LDL, low density lipoprotein; PAS, Periodic acid-Schiff; PS, phosphatidylserine; FA, fatty acids; TG, triglycerides; Cer, ceramide; PG, phosphatidylglycerol; CerG, hexosylceramide; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; C18SA, C18 sphinganine; C18SO, C18 sphingosine; doxSA, 1-deoxysphinganine; doxSO, 1-deoxysphingosine.

Abstract

Chronic kidney disease (CKD) leads to deep changes in lipid metabolism and obvious dyslipidemia. The dysregulation of lipid metabolism in turn results in CKD progression and the complications of cardiovascular diseases. To obtain a profound insight into the associated dyslipidemia in CKD, we performed lipidomic analysis to measure lipid metabolites in the serum from a rat 5/6 nephrectomy (5/6 Nx) model of CKD as well as in the serum from CKD patients. HK-2 cells were also used to examine oxidative stress-induced sphingolipid changes. Totally 182 lipid species were identified in 5/6 Nx rats. We found glycerolipids, total free fatty acids, and sphingolipids levels were significantly upregulated in 5/6 Nx rats. The atypical sphingolipids, 1-deoxysphingolipids, were significantly altered in both CKD animals and human CKD patients. The levels of 1-deoxysphingolipids directly relevant to the level of oxidative stress in vivo and in vitro. These results demonstrate that 1-deoxysphingolipid levels are increased in CKD and this increase directly correlates with increased kidney oxidative stress.

Keywords: chronic kidney disease; 1-deoxysphingolipid; free fatty acid; MDA; oxidative stress

1 Introduction

As an important public health problem, chronic kidney disease (CKD) damages up to 13% adults in America [1] and worldwide [2]. The classic biomarker used in clinics to monitor kidney function is serum creatinine, which is used to estimate the glomerular filtration rate and monitor urine output [3]. Creatinine is, however, insensitive for kidney injury, especially in the beginning process of disease [4], and provides no information on the cause and nature of renal injury, since the levels of creatinine are influenced by non-renal factors, including age, sex, nutritional status, infection, and medications [5, 6]. Large efforts have been put into identification of new markers that are more specific to renal tubular oxidative stress or can predict ''subclinical'' renal damage before serum creatinine elevation rather than assessing glomerular filtration as an endpoint.

Lipids are small molecules representing products or intermediates of metabolic processes. Lipids play significant roles in cytotoxicity, signal transduction, and energy production. The balance of lipids intake and production, transport and excretion maintains the concentrations of various lipids in body fluids and cells. Kidney plays a central role in all these processes, which provides a theoretical basis for the study of lipidomics in nephrology [7]. Recently, increasing researches indicated that serum lipid profile and lipid metabolism altered markedly during CKD pathogenesis of in vivo and in vitro [8, 9] and linked them to enhanced reactive oxygen species production [10, 11]. Lipidomics has been used to determine the lipid distribution of low density lipoprotein (LDL), which demonstrated that triacylglycerides are significantly increased, and plasmenyl ethanolamines, cholesterol sulfate, phosphatidylcholines, ceramides, and sulfatides are significantly reduced in the advanced CKD patients [12].

Sphingolipids are a class of structurally highly diverse lipids that are fundamental components of eukaryotic cell membranes. Sphingolipid de novo synthesis starts with the formation of the long chain sphingoid base, which is the characteristic backbone of all sphingolipids. This first and rate-limiting step is catalyzed by the enzyme serine-palmitoyltransferase (SPT), which typically conjugates L-serine and palmitoyl-CoA [13]. Moreover, SPT shows an alternative activity with L-alanine, which results in the formation of an atypical category of 1-deoxysphingolipids [14]. Due to the lack of C1-hydroxyl group of sphinganine, 1-deoxysphingolipid can neither form complexes (sphingomyelins and glycosphingolipids) at its head group, nor proceed for further degradation [15]. Abnormal 1-deoxysphinglipid levels are found to be associated with mutations in SPT [16], certain metabolic conditions [17-19] or reductions of cytochrome P450 enzymes [20]. However, the changes of serum 1-deoxysphingolipids in CKD, and the underlying mechanism is still unknown.

In this study, we performed a common procedure to construct Sprague-Dawley rats of 5/6 Nx-induced CKD model to assess the changes of the associated serum sphingolipid levels. To this end, we analyzed serum sphingolipid levels, renal function, and renal oxidative stress markers in 5/6 Nx rats. In addition kidney tissue and serum sphingolipids were examined in CKD patients. Both animal and clinical data indicate that oxidative

stress marker malondialdehyde (MDA) co-relates with the increase of 1-deoxysphingolipids in CKD. By using free fatty acid (FFA)-albumin overload-induced oxidative stress models in vivo and in vitro, oxidative stress is shown to mediate the increase of 1-deoxysphingolipids, independent from SPT-related de novo synthesis.

2 Materials and Methods

2.1 Animals

Male Sprague-Dawley rats (200–220 g per rat) from Vital River Laboratories (Beijing, China) were randomly divided into 5/6 nephrectomy (5/6 Nx) group and normal control sham group. Each group of rats were placed in the animal facility with a 12:12-hour day and night cycle. The animals were allowed ad libitum to regular food (AIN93M, Keao Xieli feed co. LTD, Beijing China) and water. As described previously [21], animals from CKD group received a two-stage procedure of 5/6 nephrectomy with lateral incision to expose the retroperitoneal kidney. The animals from sham-operated group received the same incision and kidney exposure, while the kidney was kept intact. For the lipidomics analysis, 5 animals per group were used. In another experiment, at least 6 animals were used in each group to confirm lipidomics results and for further analysis. All rats were sacrificed under anesthesia 8 weeks after the second stage of surgery and kidneys were harvested. Half of the kidney from each animal was snap frozen in liquid nitrogen and stored at − 80 °C for RNA extraction. The other half was fixed with formalin for histology. For FFA-induced kidney damage experiment, a mild albumin overload kidney damage model was used according to previous studies [11, 22-25], with modifications. Briefly, a 30% BSA/saline solution was made. For palmitate-loaded BSA, 150 μmolar palmitate acid was bound to 50ml of 30% BSA to make a 1mM BSA-FFA stock. Aliquots were stored at 4 degree until the time of injection. Groups of male Sprague-Dawley rats (200–220 g per rat) were randomly received i.p. injection with palmitate-loaded BSA (BSA-FFA, resolved in saline) or fatty acid-free BSA (vehicle, resolved in saline) at a dosage 1g BSA/200g body weight for 1 week.

2.2 Serum and Urine Samples Measurement

For 24-h urine collection, rats were allowed with free access to food and water. Urinary H_2O_2 levels and urinary creatinine concentrations were measured with the Amplex Red H_2O_2 assay kit (A12214, Invitrogen) and the creatinine assay kit (ab65340, Abcam), respectively. 12-hour fasted rats were used to obtain serum samples from their tail blood. Serum triglyceride, cholesterol, and malondialdehyde (MDA) levels were measured using the triglyceride assay kit (ETGA-200, EnzyChrom, Aachen, Germany), the Amplex Red cholesterol assay kit (A12216, Invitrogen), and the Lipid Peroxidation (MDA) Assay kit (ab118970, Abcam), respectively.

2.3 Renal Pathology Assessment and Immunostaining

In order to assess renal pathology, kidneys were embedded in paraffin wax following overnight fixation in 10% neutral buffer formalin. Fixed kidneys were sectioned into 4-μm-thick slices using a microtome (Leica, RM2235). Then Periodic acid-Schiff (PAS) staining was performed to determine the mesangial area according to the manufacturer's instruction. Masson's trichrome staining and Methenamine Silver (MS) staining were performed to assess the fibrotic area using standard protocols. Six rats were analyzed per group.

For immunostaining analysis, de-waxed paraffin sections (4-µm-thick) were hydrated, microwaved for 8-15 min in 10 mM sodium citrate (pH 6.0) for antigen retrieval, and then immunostained with a rabbit antibody against 4-hydroxynonenal (4-HNE, #ab46545, Abcam). Immunolabeled sections were then incubated with goat anti-rabbit second antibody conjugated to horseradish peroxidase and treated with the EnVision+ diaminobenzidine kit (DAB, Dako, Glostrup, Denmark) using standard protocols.

2.4 Lipid analysis

The lipidomic analysis was performed as described previously [26, 27]. Quality criteria for the identified lipid metabolites were linearity R2>0.9 and CV<20%. For analysis of the sphingoid base profile, the lipids were acid/base hydrolyzed to remove the headgroups and N-linked fatty acid as described earlier [20, 28]. Briefly, acid/base–hydrolyzed lipids were derivatized with o-phthalaldehyde (OPA) by redissolving in 75 µl of 56.7% MeOH, 33.3% EtOH, 10% H2O and 5 µL OPA working solution (990 µl boric acid [3%] + 10 µl OPA [50 mg/ml in EtOH] + 0.5 µl 2-mercaptoethanol). For liquid chromatographic separation of the sphingoid and deoxysphingoid base backbones, a C18 column was used (Uptispere 120 Å, 5µm, 125 × 2 mm, Interchim, Montluçon, France) coupled to a Transcend UPLC pump (Thermo, Reinach, BL, Switzerland). An atmospheric pressure chemical ionization source (APCI) was used to ionize the sphingoid and deoxysphingoid bases, which were then detected using a Q-Exactive hybrid quadrupole Orbitrap mass spectrometer (Thermo, Reinach, BL, Switzerland) run in full scan mode. The MS parameters used were as follows: scan range of m/z 120-1200, mass resolution of 140000, automatic gain control (ACG) target of 3.00E+06 and max injection time of 512 ms.

2.5 Patients

Kidney samples were collected in the Affiliated Hospital of Shandong University of Traditional Chinese Medicine. This study included sixty-three patients with CKD, as diagnosed according to KDIGO Clinical Practice Guideline for the Evaluation and Management of CKD. The exclusion criteria were: (a) liver, thyroid, or cardiovascular (previous myocardial infarction, heart failure) diseases; (b) type 2 diabetes mellitus history; (c) high body mass index (more than 40 kg/m2); and (d) medication intake known to affect the test parameters. The weight change of participants was less than 5 kg during the last 6 months. Samples from 31 gender- and age- matched healthy volunteers were used as normal controls. Clinical and demographic data of included patients, such as gender, age, creatinine, and cystatin C are shown in Table 1.

2.6 Cell culture

HK-2 cells were maintained in K-SFM medium (17005-042, GIBCO) supplied with bovine pituitary extract (0.05mg/ml, provided with the K-SFM kit) and human recombinant epidermal growth factor (5ng/ml, provided with the K-SFM kit), plated in culture at 37°C, 5% CO_2. For the free fatty acid (FFA) treatment, cells were first starved in 0.2% FBS/DMEM for 12h, cells were incubated by the addition of palmitate acid-bound BSA (100 µM PA). BSA was used as the vehicle. FFA-containing medium was prepared by the conjugation of palmitate acid with FFA-free BSA, as previously described [29].

2.7 Cell staining

FFA-treated HK-2 cells were fixed in 4% paraformaldehyde in PBS, and blocked with 1% BSA/PBS for 30 min following with 0.1% Triton X-100 treatment for 15 min. Then, the cells were incubated with primary antibody at 4 °C overnight. The cells were washed and incubated with secondary antibody for 1 h in the dark. After being washed, the cells were mounted with DAPI (Vector Laboratories) and visualized under a fluorescent microscope (Leica DMI6000B). For intracellular ROS detection, either plasmids encoding redox-sensitive GFR (roGFP plasmid #49435, AddGene) or CellROX green (Life Technologies) were used as previously described [30, 31].

2.8 Isolation of RNA from kidney tissue and cells and quantification of transcript levels

Total RNA was extracted with Trizol (Invitrogen, Waltham, MA). Two micrograms of total RNA were reverse transcribed using random primers and Superscript II reverse transcriptase (Invitrogen). First-strand cDNA was used as the template for real-time PCR analysis with primers (Applied Biosystems, CA) and TaqMan master mix. Data were calculated and expressed relative to levels of RNA for the housekeeping gene β-actin.

2.9 Statistics

Data are expressed as mean ± S.D. Statistical significance of differences between groups was assessed by Student's t test. PCA and heatmap visualization were performed using Metaboanalyst software. Correlation analysis was done using the concentrations of 1-deoxysphingolipids and the concentration of clinical biochemistry markers including the renal function parameters creatinine and cystatin c. GraphPad was used to perform statistical analyses.

2.10 Study Approval

Animal protocols and experiments abided by the laws of animal protection and were approved by the local institutional animal committee of Shandong University of Traditional Chinese Medicine, China. The research from human samples was performed according to the Declaration of Helsinki guidelines regarding ethical principles for medical research involving human subjects. Written informed consent was provided by each patient participated in the research. The study protocol was approved by the Scientific Ethical Committee of Affiliated Hospital of Shandong University of Traditional Chinese Medicine, China (license number SDU2017045).

3 Results

3.1 General data and histological analysis of a 5/6 Nx-induced chronic kidney disease rat model

Serum samples collected from rats from the 5/6 Nx group or sham group were first analyzed for creatinine and BUN content, the standard markers currently used to assess CKD. Significant, general increases in serum (Fig. 1 A) creatinine and (Fig. 1 B) BUN were noted in the 5/6 Nx group, indicating glomerular filtration damage. Histological examination of kidney tissue from 5/6 Nx rats showed clear signs of kidney damage in all individual, including glomeruli sclerosis, and interstitial fibrosis (Supplementary Fig. 1).

3.2 Lipid abnormalities in the 5/6 Nx rat model

Next, lipidomic profiling of serum was performed. The PCA score plot showed a clear separation between 5/6 Nx and sham (Fig. 1 C). 182 (10 down + 172 up) lipids passed the cut-off (1.5-fold change, $p<0.02$, and FDR<0.05) were identified (Fig. 1 D, and supplementary Table S1 and supplementary Table S2). Furthermore, there were also significant differences in lipid subclass totals in the CKD group compared to the sham group (Fig. 1 E). Lipids that were significantly increased belong to four lipid classes: free fatty acids (FFA), triglycerides (TG), sphingolipids (including ceramides, Cer; hexosylceramides, CerG), and phosphatidylglycerol (PG). These results confirm that there are profound disturbance of fatty acid and triglyceride metabolism in rats with CKD, as previously reported in CKD patients and animal models [32, 33]. Validation of serum FFA and TG confirmed increased levels of both (Fig. 1 F and G). The increase of sphingolipids in serum of 5/6 Nx rats was particularly of note. To examine changes in the sphingoid base profile, a targeted liquid chromatography-mass spectrometry analysis of the sphingoid- and 1-deoxysphingoid base composition was performed (Fig. 2). Extracted sphingolipids were subjected to a serial acid and base hydrolysis to release the N-linked acyl and O-linked headgroup structures. Levels of canonical C18 sphingolipids represented as C18 sphinganine (C18SA) (Fig. 2 A) and C18 sphingosine (C18SO) (Fig. 2 B), were identical between the two groups. However, the levels of the atypical 1-deoxysphingolipids, 1-deoxysphinganine (doxSA) (Fig. 2 C) and 1-deoxysphingosine (doxSO) (Fig. 2 D), were significantly increased in the serum of 5/6 Nx rats compared to the sham group.

3.3 Correlation between 1-deoxysphingolipid levels and oxidative stress in clinical samples

We next investigated whether there is a correlation between serum 1-deoxysphinganine and clinical parameters in human CKD patients. To distinguish the lipid changes in CKD caused from systemic disorders, patients with amyloidosis, diabetes, systemic lupus erythematosus, etc. were excluded from the study. Table 1 shows the clinical and demographic data of patients participated in this study. No significant differences were found in age and gender between patients with CKD and healthy controls. Serum creatinine and cystatin C were significantly higher in patients with CKD compared to the healthy controls. The kidneys from CKD patients showed signs of glomerulisclerosis, fibrosis and oxidative stress (Fig. 3 A). Blood levels of MDA, C18SA, doxSA, and doxSO were also increased in the CKD group (Fig. 3 B).

The relationships between serum concentrations of C18-sphingolipids or 1-deoxysphingolipids and the oxidative stress marker MDA and glomerular filtration markers (creatinine and cystatin C) in healthy subjects and CKD patients are illustrated in Table 2. Serum C18 sphingolipids were not co-related with either MDA or glomerular filtration markers (Table 2 and Figure 4 A and B). Both 1-deoxysphinganine and 1-deoxysphingosine showed significant positive correlations with MDA in CKD patients but not in healthy subjects (Table 2 and Figure 4 C and D). There was a significant negative correlation between serum 1-deoxysphinganine and Cystatin C (Table 2). There were no significant negative correlations between serum 1-deoxyshingolipids with creatinine. Interestingly, when the creatinine levels were translated into eGFR, serum 1-deoxysphingosine was positively correlated with eGFR levels in CKD patients. This indicates a positive relationship between the extent of oxidative stress and serum 1-deoxysphingolipid levels, but not with canonical C18-sphingolipids in CKD patients.

3.4 Increased serum 1-deoxysphingolipid levels are a marker of FFA-induced tubule oxidative stress in a chronic kidney disease rat model

Since fatty acids are the preferred energy source for proximal tubular cells, excessive fatty acid supply induces oxidative stress and mitochondrial dysfunction, which result in reduced fatty acid oxidation and abnormal lipid metabolism [8]. In human CKD and an animal model of 5/6 Nx-induced CKD, increased urinary albumin and albumin-bound FFA may increase the exposure of FFA to proximal tubules and result in tubular damage, oxidative stress, and mitochondrial dysfunction [11, 34, 35]. To evaluate if increased 1-deoxysphingolipid levels are a result of increased FFA exposure and subsequent abnormal lipid oxidation and oxidative stress in proximal tubules, rats were i.p. injected with BSA-bound FFA. Urinary H_2O_2 and serum levels of MDA were significantly increased by 1.5 to 2-fold in BSA-FFA compared to vehicle littermates (Fig. 5 A and B). A significant increase of lipid peroxidation was detected in BSA-FFA rats as examined by immunostaining for 4-HNE, which was closely associated with the severity of proximal tubule oxidative stress (Fig. 5 C). There was also a resulting increase in both 1-deoxysphinganine and 1-deoxysphingosine but not of the canonical sphingolipids (C18SA and C18SO) in the serum of BSA-FFA group (Fig. 5 D and E). In addition, mRNA expression of SPTLC2 (Serine Palmitoyltransferase Long Chain Base Subunit 2) and SPTLC3 (Serine Palmitoyltransferase Long Chain Base Subunit 3), the two main sphingolipid de novo synthesis enzymes, were not changed between treatment groups (Fig. 5 F and G), indicating that increased 1-deoxysphingolipids are not a direct conversion from fatty acids.

3.5 Oxidative stress induces 1-deoxysphingolipid levels in vitro

To examine whether increased oxidative stress causes the induction of 1-deoxysphingolipid levels in vitro, HK-2 proximal tubule cells were treated with palmitate acid-bound BSA. The intracellular levels of oxidative stress, indicated by CellROX or roGFP probe, were significantly induced by FFA treatment (Fig. 6 A, B and C). The oxidative stress induced by FFA resulted in an induction of 1-deoxysphingoid bases but not of the canonical C18 sphingoid bases in HK-2 cells (Fig. 6 D and E). In consistent with in vivo data, the mRNA expression of SPTLC2 and SPTLC3 were not changed between treatment groups (Fig. 6 F).

4 Discussion

Several lipid classes play an important role in biomarker research and risk stratification of cardiovascular disease [36], diabetes [37], and kidney disorders [38-40]. The lipid categories associate with biomarkers in CKD including glycerolipids, fatty acids, sphigolipids and sterols. Our results confirm that there are profound disturbance of triglyceride and fatty acid metabolism in patients with CKD, as previously reported in animal models and CKD patients [32, 33]. The present study demonstrated that serum concentrations of the atypical 1-deoxysphingolipids were elevated in CKD patients compared to healthy subjects. Furthermore, the levels of serum 1-deoxysphingolipid correlated with oxidative stress marker MDA levels in CKD patients.

Elevated intracellular levels of reactive oxygen species (ROS) play a major role in the pathogenesis of CKD [41]. To avoid cellular damage, normal kidney cell function relies on the optimal amount of ROS. Extra intracellular levels of ROS could cause DNA, lipids, and proteins oxidation. Oxidative stress is a common feature in CKD patients [42, 43], and associated with disruptions in lipid metabolism that results in upregulated glycerophosphoethanolamines and bile acids levels [44]. The important role of oxidative stress in CKD is well recognized, while there are limited clinical tools to monitor oxidative stress. Levels of the oxidative stress markers methionine sulfoxide, methionine sulfoxide-to-methionine ratio [45, 46], and malonedialdehyde [47] have already been analyzed by metabolomics. It is very difficult for quantitative analysis of oxidative stress in order to meet the special conditions for pre-analysis. Oxidative stress in CKD was demonstrated to be associated with marked alteration of serum concentrations of different lipids [32, 48]. In the present study we found that FFA-induced oxidative stress results in increased 1-deoxysphingolipid levels both in vivo and in vitro. Furthermore, the correlation between 1-deoxysphingolipid levels and oxidative stress marker, MDA, was significant in serum from CKD patients. Given the nature that 1-deoxysphingolipids are hardly degraded in the body once it is synthesized as it can not be phosphorylated by sphingosine kinase and degraded by S1P lyase, serum 1-deoxysphingolipids could be used as a useful marker for kidney oxidative stress in CKD patients.

A number of uremic toxins (including fatty acids) are found to be increased in CKD and are potent oxidative stress inducers through impairing mitochondrial function [49-51]. Considering the large number of uremic toxins, 1-deoxysphingolipids could contribute partially to oxidative stress, as examined by recent publications [28, 52]. However, several key points indicate that the increases of 1-deoxyphingolipid levels in CKD are independent to oxidative stress: (1) Because of the absence of the C1 hydroxyl group, 1-deoxysphingolipids can neither be converted to sphingomyelin through sphingomyelin phosphodiesterase (SMase), nor be degraded through sphingosine kinase (SPHK) [53]. Although increase of oxidants regulate enzyme activities of SMase [54, 55] and SPHK [56, 57] and their metabolites [58], such enzymatic pathways are not related to 1-deoxysphingolipid production; (2) A number of reports suggest that lipid accumulation in kidney could be harmful and is referred to as lipotoxicity [59]. It is still under debate whether lipids per se is are toxic, but it is clear that intra-renal lipid accumulation can cause characteristics of renal oxidative stress status [60]. The reason why 1-deoxysphingolipids are increased in CKD could be due to the dyslipidemia-induced oxidative stress and defective mitochondrial fatty acid oxidation which oftenly occurs in CKD [61, 62], resulting in an accumulation of intracellular fatty acids; (3) Among patients with diabetic nephropathy, hypertensive nephropathy, and chronic nephritis, serum levels of serine and glycine are decreased compared with healthy controls [63]. These results indicate that serine deficiency increases lipid accumulation in ESRD patients. Serine deficiency is also linked with altered glutathione metabolism and mitochondrial dysfunction (oxidative stress), as well as increased 1-deoxysphingolipid de novo synthesis [64]; (4) In parallel, reduced expression of cytochrome P450 genes and gene products (i.e., reduced mRNA and protein, or reduced protein with no change in mRNA) in several animal models of CKD were observed [65]. Although the precise mechanism(s) of the down-regulation of CYP genes in these CKD models remains unknown, ex vivo studies showed that uremic human serum obtained from patients with end-stage renal disease led to a decrease in protein expression and

activity for all the major xenobiotic-metabolizing CYPs. Such decreased cytochrome enzymes may result in reduced degradation of 1-deoxysphingolipids, which was demonstrated in our previous study in NAFLD [20, 28]; Taken together, dyslipidemia- induces oxidative stress and in parallel de novo synthesis of 1-deoxysphingolipids due to fatty acid accumulation and serine deficiency which is often seen in CKD. Furthermore, decreased cytochrome P450 enzymes may result in less degradation of 1-deoxysphingolipids and accelerate the progression of CKD through the direct lipotoxic effects of 1-deoxysphingolipids on renal cells.

The reason for increased circulating 1-deoxysphigolipids in CKD still remains unclear. When an uncontrolled influx of free fatty acids were flowing into the proximal tubules present in massive proteinuria, FFA not only promote oxidative stress and nephrotoxicity, but could be also transported to the ER where they could be used for de novo 1-deoxysphingolipid synthesis [66, 67], which may in turn increase levels of 1-deoxysphingolipids in the blood. Dyslipidemic patients benefit from the 1-deoxyshingolipids-lowering effects of fenofibrate treatment [68], which is also commonly used in the treatment against CKD. Several recent clinical studies, including subanalyses of the FIELD (the Fenofibrate Intervention and Event Lowering in Diabetes Study) and DAIS (Diabetes Atherosclerosis Intervention Study) trials, support the urinary albumin-lowering effects of fenofibrate [69-71]. However, as fibrates can be associated with a rise in blood creatinine levels, their effect on oxidative stress-related renal dysfunction is less obvious. Furthermore, although both C18 sphingolipids and 1-deoxysphingolipids could both be de novo synthesized from palmitate acid, changes of 1-deoxysphingolipid levels seem to be independent to C18 sphingolipids and de novo synthesis through SPTs, because (1) myriocin, a potent inhibitor of sphingolipid de novo synthesis, has no effects on 1-deoxysphingolipid de novo synthesis [72]; and (2) FXR, a nuclear receptor which regulates hepatocyte lipid homeostasis, induces 1-deoxysphingolipids degradation but does not change C18 sphingolipid levels [73]. Great efforts are still needed to further determine the exact molecular signaling mechanisms underlying the elevation of circulating 1-deoxysphingolipid levels.

The relations between glomerular filtration and increased serum 1-deoxysphingolipid levels are still not clear. In present study, doxSA is negatively correlated with Cystatin C, however, doxSO is positively correlated with eGFR. Since sphingolipids are constituents of lipoproteins which are too large to pass the glomeruli [74], the elimination way of sphingolipids could be different to Cystatin C, which makes it difficult to directly compare the relationships between those parameters. Furthermore, since eGFR also depends on gender, age and ethnics, the observed corrections could also be indirect. Further studies regarding to CKD stages, real GFR (assessed from clearance measurements), and other factors, such as gender and age, need to be done.

Collectively, our experimental model indicates that the increased serum level of 1-deoxysphingolipids reflects a stress, such as FFA overload or protein-bound FFA-induced oxidative stress, on the proximal tubules. The clinical results support this hypothesis and show that serum 1-deoxysphingolipids might be useful clinical markers for CKD. These results provide a novel possibility for applying for the clinical implications of sphingolipid levels in the oxidative stress status of CKD.

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Table and figure legends

Table 1. Clinical and demographic data of included patients

Table 2. 1-deoxysphingolipids significantly associated with serum oxidative stress marker MDA

Figure 1. Lipidomics analysis of serum lipids from sham and 5/6 Nx rats. **(A)** Serum creatinine and **(B)** serum BUN from sham and 5/6 Nx groups. n = 6 /group. Data are mean ± SD, student's t test. **(C)** Principal component analysis (PCA) score plot of lipidomics profiles of the two groups (n = 5 /group). **(D)** Volcano plot identified lipid species passing the cut-off (1.5-fold change and $p<0.02$). **(E)** Heatmap of clustered lipids from sham and 5/6 Nx rats. Serum levels of **(F)** fatty acids (FA) and **(G)** triglycerides (TG) from different groups. n = 6 /group. Data are means ± SD, student's t test. PS, phosphatidylserine; FA, fatty acids; TG, triglycerides; Cer, ceramide; PG, phosphatidylglycerol; CerG, hexosylceramide; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin.

Figure 2. Sphingoid base profile of total serum sphingolipids. Quantitative analysis of serum (**A**) C18SA, (**B**) C18SO, (**C**) doxSA, and (**D**) doxSO from sham and 5/6 Nx groups. The profile reflects the sphingoid base composition of serum sphingolipids measured after hydrolysis. C18SA, C18 sphinganine; C18SO, C18 sphingosine; doxSA, 1-deoxysphinganine; doxSO, 1-deoxysphingosine. n = 6 /group. Data are means ± SD, student's t test.

Figure 3. CKD patients show higher oxidative stress and increased 1-deoxysphingolipid levels. (**A**) Representative images showing (a and d) Methenamine Silver (MS) staining, (b and e) Periodic acid–Schiff (PAS) staining and (c and f) immunostaining for 4-HNE on renal biopsies from (a, c, and e) normal and (b, d, and f) CKD patients (scale bar 20 µm for MS staining and PAS staining; scale bar 100 µm for 4-HNE staining). (g to i) Quantitative analysis of (g) MS staining, (h) PAS staining, and (i) immunostaining for 4-HNE. (**B** to **F**) Violin plot analysis showing the mean and variance differences in serum (**B**) MDA, (**C**) C18SA, (**D**) C18SO, (**E**) doxSA, and (**F**) doxSO between healthy subjects and CKD patients. C18SA, C18 sphinganine; C18SO, C18

sphingosine; doxSA, 1-deoxysphinganine; doxSO, 1-deoxysphingosine. Data are means ± SD, student's t test.

Figure 4. 1-Deoxysphingolipid levels correlated with oxidative stress marker MDA. Correlation analysis between serum MDA and serum (**A**) C18SA, (**B**) C18SO, (**C**) doxSA, and (**D**) doxSO in CKD patients. C18SA, C18 sphinganine; C18SO, C18 sphingosine; doxSA, 1-deoxysphinganine; doxSO, 1-deoxysphingosine.

Figure 5. FFA overload in vivo induces oxidative stress and results in increased serum levels of 1-deoxysphingolipids. Quantitative analysis of (**A**) urinary H_2O_2 and (**B**) serum MDA from rats after vehicle (BSA) and FFA (BSA-bound FFA) injections. n = 6 /group. Data are means ± SD, student's t test. Representative images showing (**C**) immunostaining for 4-HNE on renal sections from (a) vehicle and (b) FFA groups (scale bar 50 μm). (c) Quantitative analysis of immunostaining for 4-HNE. (**D** and **E**) Quantitative analysis of serum (**D**) C18 sphingoid bases, and (**E**) deoxysphingoid bases from vehicle and FFA groups. C18SA, C18 sphinganine; C18SO, C18 sphingosine; doxSA, 1-deoxysphinganine; doxSO, 1-deoxysphingosine. n = 6 /group. Data are means ± SD, student's t test. (**F** and **G**) mRNA level of (**F**) SPTLC2 and (**G**) SPTLC3 in the kidney of rats with different treatments. n = 6 /group. Data are means ± SD, student's t test.

Figure 6. FFA overload in vitro induces oxidative stress and results in increased intracellular levels of 1-deoxysphingolipids. (**A**) Representative images of (a and b) CellROX staining and (c and d) cytosolic ROS sensing in HK-2 cells after different treatments (scale bar 100 μm). (**B** and **C**) Quantitative analysis of intracellular (**B**) ROS, and (**C**) roGPF from vehicle and FFA groups. n = 6 /group. Data are means ± SD, ANOVA test. (**D** and **E**) Quantitative analysis of intracellular (**D**) C18 sphingoid bases, and (**E**) deoxysphingoid bases from vehicle and FFA groups. C18SA, C18 sphinganine; C18SO, C18 sphingosine; doxSA, 1-deoxysphinganine; doxSO, 1-deoxysphingosine. n = 6 /group. Data are means ± SD, student's t test. (**F**) mRNA level of SPTLC2 and SPTLC3 in the HK-2 cells with different treatments. n = 6 /group. Data are means ± SD, student's t test.